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I, DAVID DANIEL CLARKE , ASSISTANT DIRECTOR PATENT SERVICES, hereby certify that the annexed are true copies of the Provisional specification and drawing(s) as filed on 23 October 1995 in connection with Application No. PN 6161 for a patent by LIONS EYE INSTITUTE filed on 23 October 1995.

I further certify that the annexed documents are not, as yet, open to public inspection.

PRIORITY DOCUMENT

WITNESS my hand this Twenty-Eighth
day of October 1996

DAVID DANIEL CLARKE
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PROVISIONAL SPECIFICATION
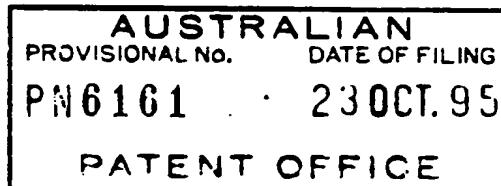
Invention Title: METHOD AND COMPOSITION FOR TREATMENT
OF OCULAR DISEASES

The invention is described in the following statement:

METHOD AND COMPOSITION FOR TREATMENT OF OCULAR DISEASES.

This invention relates to a method and composition for treating ocular diseases, in particular retinal disease involving neovascularisation of the choroid
5   and/or retina. It makes use of the phagocytic characteristic of specific cells in the eye to provide an effective manner of delivering an active agent to the target, for either short term or long term treatment of neovascularisation. The methods and compositions of the
10   invention are useful for delivering DNA, RNA, anti-sense nucleotides, peptides or other therapeutic agents to phagocytic cells or surrounding cells.

BACKGROUND OF THE INVENTION

A variety of ocular diseases such as macular
15   degeneration and diabetic retinopathy are characterised by neovascularisation of the choroid and/or retina. This process is the major cause of blindness in patients suffering from these conditions.

PRIOR ART TREATMENTS

20   In age-related macular degeneration (ARMD), the formation and haemorrhaging of a subretinal neovascular membrane (SRNVM) results in rapid and substantial loss of central vision. Various treatments which are available, but all are unreliable. Laser photocoagulation is the most
25   acceptable type of treatment, but it still suffers from the disadvantages that damage by the laser rays causes dense, permanent scotoma (Schachet, 1994; Ibanez et al, 1995 and Hudson et al, 1995) resulting in temporary loss of vision, and inability to prevent progression of the condition in
30   the long term because of recurrence of the neovascular membrane.

Thus this treatment provides an advantage only in terms of preventing profound visual loss.

Similarly, surgical removal of the SRNVM or of subretinal blood, or re-positioning of the fovea by rotating the retina have largely been unsuccessful, due to post-surgical complications and to minimum or temporary

5   improvement in vision. These invasive forms of treatment and the corresponding complications therefore far outweigh the advantages gained, and are limited in usefulness.

Administration of Interferon α2a, which has some anti-angiogenic activity (Fung, 1991; Guyer et al, 1992 and

10   Engler et al, 1994) and transplantation of retinal pigment epithelial (RPE) cells (Algvere et al, 1994) have also proved to be of limited usefulness, and initial promising results obtained with small groups of patients have not been confirmed in larger trials.

15   In addition to laser photocoagulation which, as described above, suffers from various disadvantages, the other main method of treating diabetic retinopathy is the control of blood glucose and blood pressure. The efficacy of such forms of treatment is limited by the motivation and

20   compliance of the patient involved.

About 30% of the population above age 75 suffers from macular degeneration, and about 3 in 1000 individuals suffer from diabetic retinopathy. As each of these numbers will increase due to the aging of the population, and the

25   increase in incidence of diabetes, there is a need for a more effective manner of treating these and other ocular diseases mediated by neovascularisation.

MECHANISM OF NEOVASCULARIZATION

Vascular endothelial cell growth factor (VEGF) is

30   a dimeric, disulphide-bridged glycoprotein which is well-known to be synthesised and secreted by a variety of normal as well as tumour cells. Recent observations indicate that VEGF is frequently detected in the neovascular retinal membranes of patients with diabetes (Malecaze et al, 1994),

35   the ocular fluid from patients with either diabetic

retinopathy or with central retinal vein occlusion (Aiello
et al, 1994). More recently, it was found that VEGF
expression was induced in conditions such as central vein
occlusion, retinal detachment and intraocular tumours. In

5   a rabbit model, levels of VEGF mRNA were elevated in the
hypoxic region of the retina following induction of retinal
vein occlusion. (Pe'er *et al*, 1995). Stimulation of VEGF
expression by hypoxia has also been observed in other
animal models (Pierce *et al*, 1995; Miller *et al*, 1994), and

10   *in vitro* in all types of cell cultures (Simorre-Pinatel *et*
al, 1994; Hata *et al*, 1995 and Thiema *et al*, 1995).

ANTI-SENSE DNA AND GENE THERAPY

The suppression of expression of genes encoding
proteins which mediate undesirable activity has been

15   achieved in a variety of situations by the introduction of
'anti-sense' DNA sequences into the DNA of target cells.
These anti-sense sequences are DNA sequences which, when
transcribed, results in synthesis of RNA whose sequence is
antiparallel to the sequence encoding the protein. Such

20   anti-sense sequences have been tested in a number of viral
diseases. Alternatively, anti-sense oligodeoxynucleotides
can be introduced into target cells; such short sequences
are not themselves transcribed, but inhibit transcription
and/or subsequent translation of the corresponding sense

25   DNA sequence in the target cell.

In Australian Patent Application No. 75168/94
(HYBRIDON INC), it was shown that *in vitro* expression of
murine VEGF could be inhibited in COS-1 or NB41 cells by
incubation with 19- to 21-mer anti-sense oligonucleotides

30   based on murine VEGF. A 21-mer antisense nucleotide
targeted against the translational stop site was shown to
be the effective sequence. There is no disclosure or
suggestion of specific targeting of sequences to any tissue
in the eye, or of treatment of any ocular conditions other

35   than diabetic retinopathy.

In U.S. Patent No. 5,324,654, a method of stimulating proliferation of non-malignant cells is disclosed. The method comprises the *in vitro* treatment of cells with an anti-sense nucleotide corresponding to the
5 retinoblastoma (Rb) gene to inhibit expression of the Rb gene product, resulting in suppression of the expression of proteins which inhibit cell growth. In this way, proliferation of cells is encouraged. The proliferated cells can then be re-implanted if desired, and the cells
10 may be genetically engineered to replace a specific gene prior to re-implantation. However, there is no reference to use of this anti-sense sequence to treat conditions of the eye. The invention of US5324654 is directed to establishing cell lines capable of long-term proliferation
15 and to treatment of conditions such as muscular dystrophy and diabetes, caused by failure to express a gene.

However, successful use of anti-sense nucleotides to counter expression of a gene *in vivo* is limited by factors such as the need for co-suppression of mutant gene
20 expression (Milan, 1993; McInnes and Bascom, 1992), or the need for high concentrations of the anti-sense nucleotides (Akhtar and Ivinson, 1993).

Attempts to increase uptake of anti-sense sequences into the target cell by encapsulating these
25 sequences in liposomes have been largely unsuccessful.

Another manner in which the targeting may be achieved is the employment of virus-mediated DNA transfer, using viruses such as the Sendai virus. Sendai virus is an RNA virus which has been shown to deliver DNA and proteins
30 into cells with more than 95% efficiency (Kaneda *et al*, 1987). In this gene transfer system, DNA nuclear protein complex in liposomes is directly introduced into the cytoplasm of the cell by the fusion activity of Sendai virus. The DNA is delivered rapidly into the nucleus with
35 nuclear protein. Sendai virus-mediated gene transfer occurs by fusion of the virus with the cell membrane, and

bypasses th endocytic pathway. Recently, highly efficient delivery of anti-sense or plasmid DNA into target cells by Sendai virus having been observed. Both the anti-sense and plasmid DNAs retained their activity not only in culture

5   but also *in vivo* (Kaneda *et al*, 1987). However, the use of this virus is limited by the fact that there are no suitable constructs available at present to use as vectors. In addition, the transferred DNA can only be expressed for a limited period of time since the gene transfer is

10   mediated by fusion.

Retroviruses have been widely used for somatic tissue gene therapy (Boris-Lawrie and Temin, 1993). They can target and infect a wide variety of host cells with high efficiency, and the transgene DNA integrates into the

15   host genome. Theoretically, the integration of the DNA will provide permanent production of the transgene which could result in permanent rescue of the cells. However, retroviruses cannot infect non-dividing cells (Salmons and Günzburg, 1993). Furthermore, the retrovirus particles are

20   unstable *in vivo*, which makes it difficult to achieve high virus titre with inoculation. In addition, there are significant concerns regarding the oncogenicity of the integrated viruses. The inability of retroviruses to infect non-dividing cells means that they cannot be

25   selected as candidates for gene transfer in the eye, as the most important target cells such as photoreceptors and RPE cells are non-dividing cells.

The usefulness of herpes simplex virus vectors has been limited by their poor efficiency of infection

30   (Culver *et al*, 1992). Two types of vectors have been developed, namely the replication defective recombinants and the plasmid-derived amplicons. The latter requires a helper virus. Although the toxic genes can be removed from the herpes simplex virus with difficulty, the constructs

35   remain cytotoxic (Johnson *et al*, 1992). In addition, the long term expression of the sequences inserted has been

unsuccessful to date and there are problems with th
regulation and stability of the constructs. The
application of modified herpes simplex viruses to the eye
in gene therapy poses major concerns because of their
5   pathogenicity. Herpes zoster virus infection causes
serious infections in the eye, frequently resulting in
blindness requiring corneal transplantation.

Adenoviruses have been widely used for gene
transfer in both non-dividing and proliferating cells.
10   They can accommodate DNA up to 7.5 kb, and provide
efficient transfection and high viral titre. The main
advantage of using these rather than retroviruses is the
ability to infect a wide range of non-dividing target cells
(Kozarsky and Wilson, 1993). Replication-defective
15   adenoviruses are considered to be relatively safe, in that
these viruses are common pathogen in humans, usually
causing relatively benign conditions such as colds. The
vectors carry tumour genes with a deletion mutation,
lowering the possibility of becoming oncogenic (Siegfried,
20   1993). In the first experimental gene therapy trial
approved by the NIH (National Institutes of Health, USA)
Recombinant DNA Advisory Committee, recombinant
adenoviruses were used to treat individuals suffering from
cystic fibrosis.

25   The main disadvantage of adenoviruses is their
transient gene expression. Few attempts at gene delivery
to non-dividing cells have been successful. The first
successful gene transfer into the brain, which consists of
non-dividing cells, was reported in 1993 using adenovirus s
30   (Le Gal La Salle et al, 1993).

For treatment of ocular diseases, in which other
sites in the body are largely or entirely unaffected, it is
highly desirable to deliver the therapeutic agent,
selectively to the target tissue in the eye. For anti-
35   sense DNA, it is essential that the DNA be actually taken
into these target cells.

These advances in gene therapy have led to further studies of the delivery and expression of transgenes into target cells such as beta-galactosidase transgene into the retina (Bennett *et al*, 1994, Li *et al*,
5   1994 and Mashmour *et al*, 1994) using recombinant adenovirus as a delivery system. High levels of transgene expression within 3 days in the RPE layer and within two weeks in the photoreceptor cells of the neural retina in young animals were observed. The expression of the reporter gene was
10   followed up to 9 weeks. In older animals, neither subretinal nor intravitreal injections induced the expression of beta-galactosidase transgene in the photoreceptor cells (Li *et al*, 1994).

Australian Patent Application No. 61444/94 shows
15   the uptake of replication-defective recombinant adenovirus by various tissues in the eye following injection into the anterior chamber, the vitreous humour, or the retrobulbar space, and that the reporter gene β-galactosidase is expressed. However, this document does not show that such
20   forms of viruses successfully incorporate the active agent into the target cell or area. Nor is there any disclosure or suggestion that VEGF can be used to heal any ocular condition.

Another known obstacle to success of using anti-
25   sense nucleotides as a form of therapy for the eye is the inability of the nucleotide to enter the target cells, and the limited stability of the oligonucleotides which have been modified, eg. phosphothioate oligonucleotides (Helene 1991). These factors greatly restrict the success of gene
30   therapy *in vivo*, particularly in the long term. In the treatment of retinal diseases, the ability to delay progression of the conditions by about 12 months would greatly increase the value and effectiveness of long term therapy.
35   When using adenoviruses as a transport vector for retinal gene therapy, the associated cytotoxicity has been

shown to be dose-dependant (Mashmour, 1994). In ord r to
decrease the dos of a given vector but retain its transfer
efficiency, an adjuvant may be used. Adjuvants such as
lipofectin have been shown to increase the uptake of
5   "naked" DNA by cells.

HA is a large, complex oligosaccharide consisting
of up to 50 000 pairs of the basic disaccharide glucuronic
acid-β(1-3) N-acetylglucosamine β(1-4). It is found *in
vivo* as a major component of the extracellular matrix. its
10   tertiary structure is a random coil of about 50 nm in
diameter. It has the ability to bind a large amount of
water, which *in vivo* makes it a viscous hydrated gel with
viscoelastic properties. It is found in this form in the
mammalian eye, both in the vitreous and in the
15   extracellular matrix.

The important characteristic of HA, in terms of
its acting as a potential adjuvant is its ability to bind
other molecules and to bind to cell membranes. Cell
surface receptors specific to HA have been identified,
20   including the histocompatibility antigen CD44 receptor for
hyaluroan-mediated motility (RHAMM), intercellular adhesion
factor (ICAM) and some homologous proteins in the CD44
family.

The putative adjuvant mechanism of action is
25   based on the the presence of surface receptors on many cell
types and the ability of HA to bind molecules. The binding
of virus to the cell membrane facilitated by HA would allow
the usual endocytotic mechanisms of viral uptake to be more
effective.

30   Even though HA has been widely used in eye
surgery as a replacement for vitreous humour lost during
the surgical procedure, we are not aware of any suggestion
in the art that HA promotes uptake of any pharmaceutical
agent into any cells or tissues in the eye. Similarly,
35   although HA has been suggested to promote penetration of
pharmaceutical agents such as antibiotics or anti-cancer

agents, as set out in Australian Patent Application No. 52274/93 by Norpharmco, this specification does not suggest that HA promotes uptake of any agent, let alone DNA, or viruses by individual cells of any type. In particular,

5   this invention does not teach the use of HA via intra-ocular injection.

The retinal pigment epithelium (RPE) is a non-renewable single cell layer in the eye, situated between the neural retina and the choroid. The cells of the RPE

10   are phagocytic neuroepithelial cells which form the outer most layer of the retina. The phagocytic properties of these cells have long been known, and have been reviewed (Bok and Young, 1979).

We have now found that the phagocytic nature of

15   the RPE cells enables the cells to take up large molecules such as oligonucleotides, following injection into the vitreous space *in vivo*, and that the use of replication-defective adenovirus to deliver the nucleotides confers stability to the latter. Our findings enable the induction

20   of both long term and short-term inhibition of VEGF expression in retinal or choroid epithelial cells, and hence inhibition of neovascularisation of the retina.

SUMMARY OF THE INVENTION

According to one aspect, the invention relates to

25   a pharmaceutical composition for treatment of retinal diseases mediated by abnormal vascularisation, comprising anti-sense nucleic acid sequences directed against vascular endothelial growth factor, and optionally further comprising adjuvants for increasing cellular uptake such as

30   hyaluronic acid, together with a pharmaceutically acceptable carrier or vehicle.

In separate embodiments, this aspect of the invention is directed to treatment for such retinal disease in the short term (up to about two months), the long term

35   (up to about one year), and indefinite (for the life of the

patient). In the first embodiment, for short term
treatment the invention provides one or more anti-sense
oligonucleotides having 100% complimentarity to a
corresponding region of the VEGF gene. The oligonucleotide

5   should have 16 to 50 nucleotides, preferably 16 to 22, and
more preferably 16 to 19 nucleotides.

For long term inhibition, the invention provides
a recombinant virus comprising VEGF DNA in the anti-sense
direction. This VEGF DNA is a long sequence, which for

10   purposes of this specification is to be understood to
represent a VEGF sequence of greater than 50 nucleotides in
length, ranging up to the full length sequence of VEGF. In
this embodiment, the recombinant virus is accummulated in
RPE cells, and produces anti-sense VEGF *in situ*, thereby

15   inhibiting VEGF expression in the RPE cell.   The VEGF is
most preferably human retinal pigment epithelial (RPE) or
choroidal endothelial VEGF.

In another aspect, the invention provides a
method of prevention or amelioration of retinal diseases

20   caused by abnormal neovascularisation, comprising the step
of administering an effective amount of an anti-sense
nucleic acid sequence directed against VEGF into the eye,
thereby to inhibit neovascularisation.

For indefinite inhibition, the invention provides

25   an adeno-associated or similar virus comprising VEGF DNA in
the anti-sense direction. As in the embodiment directed
for long term treatment, this VEGF DNA is of at least 50
nucleotides. The adeno-associated or similar virus
facilitates integration of anti-sense VEGF DNA into the RPE

30   cell genome, thus enabling expression of anti-sense VEGF
for as long as the cell remains functional. Eye diseases
which may be treated using the compositions and methods of
the invention include but are not limited to age-related
macular degeneration (ARMD) and diabetic retinopathy.

35   Other ocular condition and tissues in which no
vascularisation occurs (rubeosis iridis) or in the cornea

may also b treated by the invention.

The anti-sense sequence may be carried in a
replication defective recombinant virus, as a vector or
vehicle. The vector preferably comprises replication
5   defective adenovirus carrying promotes such as the
respiratory syncytial virus (RSV), cytomegalovirus (CMV),
adenovirus major late protein (MLP), or β-actin promotes.
In a particularly preferred embodiment, the vector is
pAd.RSV or pAd.MLP. In a more particularly preferred
10   embodiment the virus used is Ad.RSV.VEGF. The vector may
also comprise a polyadenylation signal sequence such as the
SV40 signal sequence.

In a preferred embodiment, human VEGF is
subcloned into the vector in order to create the
15   restriction sites necessary for insertion into the vector
to form an adenovirus plasmid carrying VEGF on partial
sequences thereof in an anti-sense direction, which can
then be linearised by restriction enzyme digestion. The
linearized plasmid which can then be co-transfected with a
20   linearized replication defective adenovirus, such as Ad.RSV
βgal recombinant virus in a suitable permissive host cell
such as the kidney 293 cell line.

The compositions of the invention may be
delivered into the eye by intra-vitreal or sub-retinal
25   injection, preferably in an appropriate vechicle or
carrier. Such methods of administration and vechicles or
carriers for such injection are known in the art.
Alternatively, *ex vivo* delivery in the compositions of the
invention may be achieved by removal of RPE cells from the
30   patient to be treated, culturing the cells and subjecting
them to infection *in vitro* with a replication-defective
adenovirus or an adeno-associated virus as defined above.
RPE cells carrying the virus are then injecting into the
sub-retinal layer of the eye of the patient.
35   While the invention is specifically described
with reference to conditions of the eye, the person skilled

in the art will be aware that there ar many other
pathological conditions in which VEGF is of importance.
Such a person will understand that the antisense
oligonucleotides and the recombinant viruses of the
5   invention are applicable to treatment of such other
conditions.

Brief Description of the Figures
       Figure 1 shows the results of GeneScan analysis
of persistance of anti-sense oligonucleotides *in vivo* in
10   the retina following a single intra vitreal injection.
       Figure 2 is a graphical representation of the
number of phagosomes in the RPE layers of Long-Evans rats.
Doses were as follows: Low 6.6 µg, medium 66 µg and high
132 µg of CatSC anti-sense oligonucleotide. Each column
15   shows the mean and standard deviation of the number of
phagosomes in five randomly selected areas in the rat
retinas; and
       Figure 3 is a graphical representation of the
number of phagosomes in the RPE layers of RCS-rdy+ rats.
20   Experimental animals were injected with 66 µg of sense
oligonucleotides (S1) and 66 µg of antisense
oligonucleotide (CatSC).

Description of the Invention
       The invention will now be described by way of
25   reference only to the following non-limiting examples. In
some of these examples, the feasibility of the methods
utilised in the invention is demonstrated using anti-sense
oligonucleotides complementary to cathepsin S (CATSC).

Example 1   Accumulation of Antisense Oligonucleotides
30   in the RPE Cell Layer
       Human retinal pigment epithelial cells were
cultured and on the third passage were used for *in vitro*
experiments. Confluent cultures were incubated with bovine

rod outer segments (ROS) to mimic the *in vivo* situation. A fluorescein-labelled anti-sense oligonucleotide complementary to human cathepsin S (CATSCF) was added to the medium of these cells and after 7 days of incubation,

5 the cells were harvested. The presence of fluorescein-labelled oligonucleotides within the RPE cells was detected by fluorocytometry (FACS). A GeneScan DNA analyser was used to assess the presence and stability of the oligonucleotides in the cells. The fluorescence of

10 cultured RPE cells was increased by about 100-fold, demonstrating the presence of the anti-sense oligonucleotides within the RPE cells. These results are summarised in Table 1

15 <div align="center">

Table 1

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Fluorocytometer measurements of human RPE cells
incubated with or without complementary CATSCF

SAMPLE	FACS READING
RPE + ROS	5.94
RPE + ROS + CATSC	8.50
RPE + ROS + CATSCF	461.50

20

It was not known if the fluorescence was emitted by the full length CATSC or by degraded oligonucleotides. Using GeneScan, it was demonstrated that the fluorescence

25 was largely due to a 19-mer oligonucleotide, which appeared at a position similar to that of CATSCF. Using a similar procedure, it was observed that CATSC oligonucleotides were still intact after 7 days of incubation.

Example 2 Cellular Distribution of Oligonucleotides
     in Retinal Cells and Stability of
     Oligonucleotides Following Injection Into
     Eyes

5   One mmole of CATSCF was injected into the
vitreous humour of 6-week old non-pigmented RCS-rdy$^+$ rats,
and the movement of the oligonucleotides were followed by
confocal fluoromicroscopy. Fluorescein (1mmole) was also
injected as a control. Animals were euthanised 2 hours, 3
10 days and 7, 14 and 28 and 56 days after injection.
Following euthanasia, the injected eyes were enucleated,
frozen, sectioned and immediately used for confocal
microscopy without fixation.

   Two hours after intravitreal injection of CATSCF
15 the penetration of the oligonucleotides were observed in
the ganglion cell layer at 2 hours and also in the
photoreceptor and pigment epithelial layers at 3 days.
However, 7 days following injection, only the RPE layer had
significant amounts of CATSCF. At 14, 28 and 56 days, a
20 fluorescent signal was maintained in the RPE layer, and no
signal was observed in any other cell types. These results
show that a large proportion of CATSCF was taken up by the
phagocytic RPE cells.

   Following intravitreal injection as described
25 above, eyes were dissected, the retina was removed, and the
DNA extracted. The purified DNA was subject to GeneScan
analysis. The presence of undegraded fluorescein-labelled
oligonucleotide was demonstrated in the rat retinas after
7, 14, 28 and 56 days of injection, as shown in Figure 1.
30 The intensity of the signal had significantly diminished by
56 days.

   These results demonstrate that following
intravitreal injection, oligonucleotides accumulate in the
RPE cells. The oligonucleotides are present in the RPE
35 layer up to 30 days and remain in a biologically active
form during this period of time.

Example 3   **Biological Activity of Anti-Sens**
                  Oligonucleotides

Female sixty day-old pigmented rats of the Long-
Evans strain were obtained from Charles River Breeding
5   Laboratories, Wilmington, MA.

Sixty day old non-pigmented RCS-rdy + rats were
obtained from our colony. The animals were acclimatised to
a 12 hr light/ 12 hr dark lighting cycle, with an average
illuminance of 5 lux for at least 10 days prior to
10   experimentation.

Animals were anaesthetised by intraperitoneal
injection of sodium pentobarbital (50 mg/kg body weight).
Intravitreal injections through the pars plana were made
using a 32 gauge needle. The left eyes served as controls,
15   and the right eyes were injected with 3μl of 150 mM sodium
chloride (saline), or with 3 μl of saline containing 6.6,
66 or 132 μg of CATSC respectively, an anti-sense
oligonucleotide described earlier (Rakoczy et al, 1994) or
66 μg of sense oligonucleotide S1, 100% complementary to
20   CATSC. Injected animals were allowed to recover from
anaesthesia, and at one week post-injection were sacrificed
by an overdose of sodium pentobarbital and used for
morphological examination. All animals were killed within
half an hour at the same time of the day, approx. 4 hours
25   after light onset. Two to three animals were used for each
dose.

Following enucleation, whole eyes were immersed
in 2.5% glutaraldehyde and 1% paraformaldehyde in 0.125M
sodium cacodylate buffer, pH 7.35. The cornea and lens
30   were dissected free and the eyecup trimmed for orientation
purposes. The tissue was fixed overnight at 4°C and then
post-fixed for 1 hour in 1% osmium tetroxide at room
temperature. After ethanol dehydration, the tissue was
embedded in epoxy resin. Retinal sections were prepared
35   for transmission electron microscopy (EM) as described
previously (Kennedy et al, 1994).

Histological data were obtained by light
microscopy. Semi-thin 1 μm sections were cut using a LKB
2088 Ultratome (LKB-Produkter, Sweden) with a diamond knife
and stained with toluidine blue. The number of phagosomes
5 that accumulated in the RPE cells of each specimen injected
with saline, low (6.6 μg), medium (66 μg) or high 132 μg)
dose of CATSC and 66 μg of S1 sense oligonucleotide was
determined. From each eye, five sets of counts were made
at 40 fold magnification and the standard deviation was
10 calculated. Each set consisted of the total number of
phagosomes in 250 μm length of RPE from 6 different
randomly selected areas. The number of phagosomes that
accumulated in the RPE of the control eyes, low medium and
high doses of CATSC were analysed and graphically
15 represented. Comparisons were made using the analysis of
variance following the general linear models procedure of
the SAS[R] (version 6) statistical package (SAS Institute
Inc., USA).

The results show that we successfully tested an
20 antisense oligonucleotide (CATSC) in two strains of rats.
The number of phagosome-like inclusion bodies present in
control Long-Evans and RCS rdy + rats was not significantly
different, 35.8+11.6 and 47.29+14.8 (mean ± SD),
respectively, The intravitreal injection was non-
25 traumatic. Light microscopic examination of the retinas of
the saline injected eyes revealed no damage to the outer
layers of the retina, and there was no increase in the
number of phagosome-like inclusion bodies in the RPE layer
when compared to the control non-injected animals. Long-
30 Evans rats were used to identify the minimum amount of
CATSC required to induce biological changes in the RPE
layer. In the control eyes and in those injected with low
dose (6.6 μg) of CATSC, the number of phagosome-like
inclusions within the RPE cells, were 35.8+11.6 and
35 35.0+7.4 respectively. In animals injected with higher
doses (66μg and 132 μg), the number of phagosome-like

inclusions were 96.2+13.6 and 141.0+34.7, respectively, and the difference was statistically significant when compared to the control and low dose samples (Figure 2).

5   RCS-rdy+ rats injected with 66 µg of CATSC also demonstrated a statistically significant increase in the number of phagosome-like inclusion bodies, ie 204.20+39.3 when compared to the 47.20+14.8 in controls. In contrast, the injection of 66 µg of sense oligonucleotide (S1) did not increase the number of phagosomes (Figure 3) present in
10   the RPE Layer, (34.4+12.54).

The inclusions found in RPEs of CATSC-injected Long-Evans and RCS-rdy+ animals were spherical in shape, and clearly distinguishable from the very dark, small elliptical melanin granules present in Long Evans rats. In
15   the presence of 66 µg of CATSC, the tips of the outer segments showed signs of disorganisation and there were some vacuoles present in the outer nuclear layer. However these changes were not observed in S1 sense oligonucleotide-injected animals.

20   Electron microscopic examination of the RPE layer of a CAT SC-injected eye revealed no significant changes in the morphology of RPE cells. Melanin granules appeared smaller and less concentrated due to regional differences. Individual mitochondrial profiles were smaller in the
25   treated group than in the controls, although the number was greater in the treated than in the untreated animals. Electron microscopic examination confirmed that the structures of the undigested material was similar to that of phagosomes (1,2,3). The numerous phagosomes seen in the
30   RPE layer of rats treated with CATSC were paranuclear, and contained mainly compacted phospholipid membranes, resembling undigested photoreceptor outer segment (POS) and confirming their photoreceptor origin. There were no other morphological changes observed in the POS layer, except for
35   the disorganised appearance of the apices in treated animals.

Example 4   **Delivery of Vehicle Molecules to the RPE Cell Layer**

       The effect of hyaluronic acid as adjuvant
However, we decided to study the effects of hyaluronic acid
(HA) on viral gene transfer and its ability to stimulate
uptake of DNA transported in an adenoviral vector were
examined.

       1% Hyaluronic acid extracted from rooster combs
(HEALON, Pharmacia) was prepared as 10 mg/ml solution. For
all experiments, HA was diluted with MEM. The moiety whose
uptake of which was to be measured using HA as adjuvant was
Ad.RSV.βGal

       The effect of chondroitin sulphate and
lipofectamine on the uptake of the above compounds was also
investigated.

       RPE or F2000 fibroblast cell cultures were grown
as described in (Rakoczy et al, 1992). They were harvested
and pooled. One ml aliquots of the pooled cell suspension
were placed into each well of a 24 well plate, to ensure
equal seeding of wells. The cells were incubated and
allowed to reach 95% confluency.

       Solutions of 0.001% and 0.002% HA were prepared
using MEM.10 µl of virus solution was added to 10 ml of
each of the diluted HA solutions and to 10ml of MEM
control, and incubated for 30 minutes at 25°C with
intermittent gentle shaking.

       One ml of each of the test solutions and control
was added to each cell of a 24 well-plate. There were four
samples for each of the test concentration and the control.
The viral solutions were incubated with the cells for
16 hours. Each experiment was terminated by removing the
medium from the well and fixing the cells with 0.5 ml of
0.5% glutaraldehyde per well. The glutaraldehyde was
removed after 5 minutes and the cells were washed once with
PBS. Following this, 0.5 ml of x-gal stain (Australan
Biosearch, Perth) was added to each well and incubated

overnight at room temperature.

Counting was carried out by a single observer. A second observer then blind counted half of the plates as a countercheck.

5   All cells staining positively with the x-gal stain were counted. For F2000 fibroblasts, the entire plate was counted at 200X magnification using a counting graticule within the microscope. For the RPE7 experiments, half of each plate was counted and the results multiplied

10   by 2.

The following doses of adjuvants were used:-

	0.05%	0.01%	0.005%	0.001%	Control	Control
5 µl	176[a]	318	319	316	279	282
10 µl	305[a]	906	802	645	623	609
25 µl	-[a]	714[b]	1682	1822	1478	1184
50 µl	-[a]	2772	2692	3328	2250	1822

[a]   The viscosity of these solutions precluded adequate dispersion of the HA and made them very difficult to manipulate.

[b]   It was not clear why this figure fell so far outside of the normal distribution of the other results.

Effect of HA as an Adjuvant on the uptake and expression of the β-gal gene using a viral vector

Experiment 1

	1	2	3	4	Average
RPE 7/HA (0.001%)	17114	20776	18730	17998	19168
RPE7/HA (0.005%)	17688	22186	20258	22236	20592
RPE 7/Cont	10782	15480	16326	15266	14705

Experiment 2

	1	2	3	4	Average
F 2000/HA (0.001%	4358	4620	Contam.	?	4489
F 2000/HA (0.005%)	4506	3914	4759	4332	4378
F2000 Cont	3884	3652	3875	3748	3789

The two-tailed student t Test was used to assess the significance of the difference between the means for each set of experimental data. For each experiment, the means, the Standard error of the differences of the means and the p value for the t Test are given. In both experiments, HA gave very significantly increased uptake ($p < 0.05$).

Example 5   Up and Down Regulation of Cathepsin D Expression in NIH 3T3 Cells

A 1620 bp HindIII fragment of human cathepsin D was subcloned into pHBApr-1-neo vector in both sense and anti-sense directions. Positive clones were selected, and

the orientation of the fragments was confirmed by EcoRI
restriction enzyme analysis. For the transfections of
NIH 3T3 cells the clones carrying cathepsin D in anti-sense
and sense direction were on caesium chloride density

5   gradients.

NIH 3T3 cells were seeded onto 6 well tissues
culture plates at a concentration of $2X10^5$ in 2 ml DMEM
supplemented with 10% fetal bovine serum (FBS). The cells
were incubated overnight at 37°C until they became 70%

10   confluent. Having reached this confluency the cells were
washed twice with serum and antibiotic-free medium.
Lipofection reagent (10 µl) (GIBCO-BRL) and 2 diluted in
100 µl of OPTI-MEM (GIBCO-BRL) were gently mixed and
incubated at room temperature for 15 minutes. Following

15   incubation an additional 800 µl of OPTI-MEM was added to
the mixture. This diluted mixture was gently overlaid onto
the washed NIH 3T3 cells. The cells were incubated for 16-
20 hrs before the transfection media was removed and
replaced with DMEM supplemented with 10% FBS. After a

20   further 48 hrs incubation the cells were trypsinised and
subcultured at 1:5 in media containing 10% FBS and
Geneticin 418 (GIBCO-BRL) at 1 ng/ml concentration.
Successfully transfected cells selected with Geneticin 418
were maintained in media supplemented with BS and Geneticin

25   418 as described earlier. Confluent transformed cultures
were frozen for storage and subcultured for further
analysis. The presence of cathepsin D in the transformed
NIH 3T3 cells were detected with polyclonal antibody
against cathepsin D, using a conventional cytochemical

30   technique using an alkaline phosphatase-labelled second
antibody.

Results

The presence of cathepsin D fragment of the
vector was demonstrated with HindIII digestion Positive

35   clones demonstrated the presence of 1620 kb fragment.   The

orientation was established by ECO RI restriction enzyme digestion, which gave two fragments at 5.7 and 5.9 kb in the case of anti-sense orientation and 4.3 and 7.3 kb in the case of sense orientation. All NIH 3T3 cells surviving

5   Geneticin 418 selection carried cathepsin D clones, which carry antibiotic resistance. The transformed control NIH 3T3 cells did not survive the selection procedure. Immunocytochemistry results suggest that NIH 3T3 cells carrying cathepsin D in the sense direction unregulated

10   cathepsin D production, while those carrying cathepsin D in the anti-sense direction down regulated cathepsin D production.

Example 6   Cloning and Characterisation of Human RPE
                 Vascular Endothelial Growth Factor (RPE-
15   VEGF)

Human RPE cells, available in our laboratory, are grown in tissue culture. To upregulate VEGF expression cell cultures are treated in hypoxic conditions. The upregulation of VEGF expression is monitored with

20   immunohistochemistry. The mRNA is extracted from 10^7 RPE cells, and a cDNA library carrying all genes expressed in the RPE/choroid is established using methods known in the art.

VEGF is a highly conserved molecule which is

25   highly homology between different species. A murine VEGF cDNA clone, available in our laboratory, is used to screen the human RPE cDNA library in order to identify the full length human RPE-VEGF clone. Positive clones are analysed by restriction enzyme analysis and finally by DNA

30   sequencing. Full length RPE-VEGF clones are analysed to elucidate their genomic structure (initiation sequences, start and stop codons, putative exons etc.).

Clones carrying the full length RPE-VEGF are analysed for the expression of VEGF protein with in vitro

35   translation. The identified clones are used to derive the

anti-sense molecule for insertion into the vehicle system
and for the selection of the anti-sense oligonucleotides.

Example 7   Pharmaceutical Agent for the Short Term
                 Inhibition of VEGF Expression

5   Anti-sense DNA technology enables the sequence
specific inhibition of target molecules without affecting
the non-targeted functions of the cell. As described
above, we have demonstrated both *in vitro* and *in vivo* that
anti-sense DNA can be used effectively to inhibit the anti-
10   sense oligonucleotide into the vitreous.
                A panel of 16-19-mer oligonucleotides, 100%
complementary to parts of the VEGF gene, is selected from
the 5' and 3' ends of the DNA sequence. Sense and
scrambled sequences are also used as control.
15   Phosphothioate-protected oligonucleotides are sythnesized
on a DNA synthesizer and subjected to purification.

Example 8   Anti-Sense Agent for the Long Term
                 Inhibition of VEGF Production
                Human VEGF is subcloned into the appropriate
20   vectors in order to create the restriction sites necessary
for insertion into p.Ad.RSV. The adenovirus plasmid
containing the human RPE-VEGF gene in the anti-sense
direction is characterised and positives are subjected to
homologous recombination.
25   The recombinant plasmid pAd.RSV.a.VEGF is
linearised Ad.RSV.bGal recombinant virus. Confluent layers
of kidney 293 cells are co-transfected with the linearised
plasmids and incubated by 7 days.
                Using blue-white colony selection white colonies
30   are picked and analysed for the presence of VEGF in anti-
sense orientation. Initially the presence of VEGF anti-
sense oligonucleotide is identified by restriction enzyme
analysis, PCR amplification followed by Northern blot
analysis looking for the expression of anti-sense VEGF RNA.

Modification of the recombinant adenovirus are performed to assess their effect on improving anti-sense VEGF DNA expression. These include removal of polyadenylation signal, change of promoter, shortening the VEGF insert.

5   Example 9   Construction of a Vehicle for the Permanent
                        Expression of Target Molecules

The vehicle described in Example 8 is suitable for long term treatment in that it provides temporary (maximum one year) expression of the anti-sense VEGF DNA
10   molecule and consequent protection against neovascularisation. To achieve indefinite treatment, we propose the application of a vector system which enables the integration of VEGF in anti-sense direction into the human genome present in RPE cells, which means that the
15   protection against neovascularisation can be provided for the rest of the life of the patient (as long as the RPE cells remain functional), using an adeno-associated virus (AAV) vector.

Adeno-associated viruses are non pathogenic, are
20   able to infect non-dividing cells, an have a high frequency of integration. We use AAV-2, which is a replication defective parvovirus which can readily infect other (RPE) cells and integrate into the genome of the host cells. Recent characterisation has revealed that it specifically
25   targets the long arm of human chromosome 19.

AAV constructs will use varying promoter sequences in combination with a reporter gene. The expression of the reporter gene mRNA may be detected with PCR amplification or in situ PCR, and the integration of
30   the reporter gene may be identified by chromosomal analysis of RPE cells.

Using the appropriate restriction sites the reporter gene is replaced by anti-sense VEGF DNA is cloned. The new construct is co-transfected with he complementing
35   plasmid (pAAV/ad) into kidney 293 cells previously infected

with adenovirus type 5 to make rAAVaVEGF construct. The
construct produced is used to infect RPE cells, and the
expression of anti-sense VEGF is detected by PCR
amplification.

5   Example 10   Model Systems for Testing Inhibition
                         In Vitro

        Human VEGF is cloned into COS cells to produce a
culture system (VEGF-COS) in which the effective inhibition
of VEGF expression can be tested. The inhibition of VEGF

10   expression is tested by Northern and Western blot analyses
and quantified by immunoassay.

        Toxicity of increasing concentration of
oligonucleotides on COS cells is assessed with the trypan
blue assay. The proliferation of COS cells is monitored

15   with or without the presence of increasing concentration of
oligonucleotides. The inhibition of the expression of VEGF
in controls and in cultures maintained in the presence of
anti-sense oligonucleotides is monitored by Northern and
Western blot analyses, immunocytochemistry and by a

20   quantitative immunoassay.

        RPE cells are cultured in hypoxic conditions and
the upregulation of VEGF expression are monitored in the
presence of increasing concentration of oligonucleotides
for an extended period of time. Toxicity, proliferation

25   assay and the monitoring of VEGF expression are performed
as described earlier.

        CEC cells are cultured in normal and hypoxic
conditions wit or without the presence of increasing
concentration of oligonucleotides. In addition to the

30   toxicity, proliferation assay and VEGF detection the affect
of anti-sense oligonucleotide mediated inhibition of VEGF
expression on tube formation is analysed. RPE/CEC dual
cultures produced in normal and hypoxic conditions will be
subjected to similar tests. The same model system are used

35   to ass ss the long term and permanent agents of the

invention.

Example 11   Inhibition of RPE-VEGF Expression with
                 Anti-Sense Oligonucleotides, Ad.RSV.aVEGF
                 and rAAVaVEGF *In Vivo* in Rats

5   Neovascularisation can be induced using pocket
implants in the choroid or the subretinal layer. One of
the disadvantages of these models is that the process of
neovascularisation might not follow the same biochemical
steps which naturally occur in humans suffering from ARMD.
10   To overcome these difficulties we use an animal model in
which choroidal neovascularisation is induced by VEGF
overexpression in the RPE cells. Using recombinant
adenoviruses carrying VEGF, for example Ad.RSV.VEGF, for
the *in vivro* trials all animal models described above are
15   utilised to provide us with a wide range of information.
Tests are conducted to demonstrate the expression of a VEGF
expression over a period of one year. Using Northern and
Western blot analysis VEGF down-regulation is monitored and
immunohistochemistry is used to demonstrate the down-
20   regulation of VEGF expression in a cell-specific manner.
Using the above described animal models, choroidal
neovascularisation is monitored by histology and
angiography. These models are applicable to all these
embodiments of the invention.

25   It will be apparent to the person skilled in the
art that while the invention has been described in the
Examples, various modifications and alterations to the
embodiments described herein may be made without departing
from the scope of the inventive concept disclosed in this
30   specification.

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following pages, and are incorporated herein by this
reference.

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<u>LIONS EYE INSTITUTE</u>   23 October 1995

Antisense Oligo Persistence In
The Retina / RPE of
Rat Eye Tissue
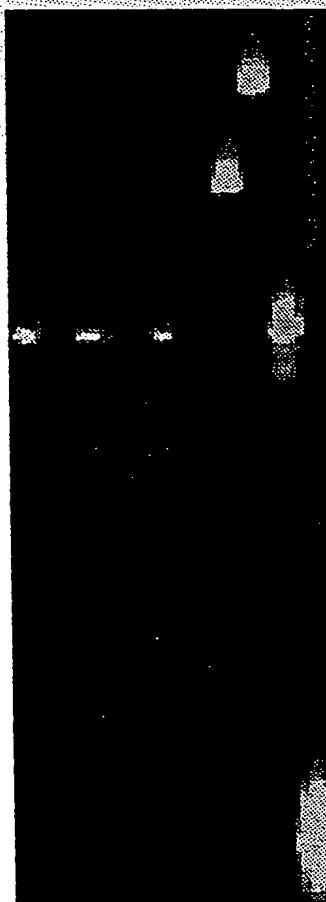
1= FAM 23 bases; 2= FAM 27 bases;
P= Antisense Primer; F= FAM Dye
I= Injected oligo; U= Uninjected

Fig. 1

Number of Phagosomes

CatSC Dose

Control

Low

Medium

High

0 20 40 60 80 100 120 140 160 180

Fig. 2

No. f phag somes

0   50   100   150   200   250

Control

S1

Treatment

CatSC

Fig. 3